Plant Physiol. (1990) 94, 1748–1755
0032-0889/90/94/1748/08/$01.00/0

Received for publication April 24, 1990
Accepted August 29, 1990

Separation and Partial Purification of 1,3-β-Glucan and 1,4-β-Glucan Synthases from *Saprolegnia*

Vincent Bulone, Vincent Girard, and Michel Fèvre*

Laboratoire de Biologie Cellulaire Fongique, Centre de Génétique Moléculaire et Cellulaire, Unité Mixte de Recherches UMR Centre National de la Recherche Scientifique 106 (bât. 405), Université Lyon I, 69622 Villeurbanne Cedex, France

ABSTRACT

Enriched 1,3-β-glucan and 1,4-β-glucan synthase fractions from the fungus *Saprolegnia* were isolated by rate zonal centrifugation on glycerol gradient. Purification was improved by entrapment of the enzymes in their reaction product, *i.e.* microfibrillar glucans. 1,3-β-Glucan synthases were separated from 1,4-β-glucan synthases following resuspension of entrapped enzymes. Sodium dodecylsulfate-polyacrylamide gel electrophoresis indicated that 1,3-β-glucan and 1,4-β-glucan synthases may have a different polypeptide composition because they were enriched for different protein subunits (34, 48, and 50 kD for the 1,3-β-glucan synthase and 60 kD for the 1,4-β-glucan synthase).

β-Glucan synthases are membrane-bound enzymes involved in cell wall morphogenesis. Over the past years, efforts have been directed towards characterization of properties and the function of these enzymes in bacteria, fungi, and green plants (see for review refs. 3 and 8).

In green plants, cell wall polysaccharides are cellulose and hemicellulose; 1,3-β-glucan synthesis occurs usually as a wound response. Activation of latent enzymes of the plasma membrane or conversion of 1,4-β-glucan synthase by moderate proteolysis could lead to the deposition of 1,3-β-glucans (3, 10). In the fungus *Saprolegnia,* 1,3-β-glucans and cellulose are integral parts of the cell wall. Isolated membrane fractions exhibit *in vitro* glycosyl transferase activities producing 1,3-β-glucan or 1,4-β-glucan synthesis according to the assay conditions (9).

By using various techniques (gradient density centrifugation, column chromatography, electrophoresis) it has been shown that glucan synthases are large protein complexes (>450 kD), and several protein subunits ranging from 18 to 83 kD might be involved in glucan synthesis (5, 16, 19).

Our previous results have shown that particulate or solubilized enzymes from *Saprolegnia* produced *in vitro* microfibrillar glucans. When these glucans sediment, they are found to be associated with particles that might correspond to the enzymes that have produced them (9). In the present paper, we report on the partial purification of 1,3-β-glucan and 1,4-β-glucan synthases. Enzymes pelleted by an entrapment procedure (12) with the reaction product were solubilized and then purified by density gradient centrifugation.

MATERIALS AND METHODS

Saprolegnia monoica Pringsheim No. 53967 Dick, obtained from CBS Baarn, the Netherlands, was grown as described previously (9). UDP-D-[^{14}C]Glucose (7.4 MBq·μmol^{-1}) was purchased from CEA, Saclay (France). Chaps[1] and UDP-Glc were from Sigma Chemical Co. All other chemicals were of the highest purity available.

Isolation of Particulate Enzymes

Membrane-bound enzymes were isolated by differential centrifugation as described previously (9).

Chaps Solubilization of Glucan Synthases

Chaps prepared in extraction buffer (0.01 M Tris-HCl, 0.5 M sorbitol, pH 7.2) was added to an equal volume of particulate enzymes at the final detergent concentration of 5 mg·mL^{-1}. Protein of the particulate enzymes were about 10 mg·mL^{-1} and were determined by the Bradford (2) assay. After vigorous vortex mixing and solubilization for 30 min at 4°C, the suspension was centrifuged at 48,000*g* for 1 h. The supernatant was used as the solubilized enzyme.

β-Glucan Synthase Assays

1,4-β-Glucan synthases were assayed in a 370-μL volume containing 0.3 nmol of UDP-[^{14}C]glucose, 0.1 μmol of DTT, 2 μmol of cellobiose, 5.5 μmol of $MgCl_2$, 25 mM Pipes-Tris buffer (pH 6.0), and 100 μL of freshly prepared enzymes. When enzymes were assayed for 1,3-β-glucan synthesis, $MgCl_2$ was omitted and UDP-[^{12}C]glucose levels increased to 400 nmol. Assays were performed in duplicate at 27°C for 30 min. Reactions were terminated by addition of 2 mL of ethanol and precipitated overnight at −20°C. After addition of carrier powdered cellulose (10–20 mg), mixtures were filtered through glass-fiber filters (Whatman, GF/C). Residues on the filter were washed with 4 mL of water, 2 mL of chloroform-methanol (2:1, v/v), and 4 mL of ethanol. Glucose incorporation into ethanol-insoluble glucans was counted by scintillation counting using a 2,5-diphenyloxazole/1,4-bis(2-[5-phenyloxazolyl]benzene toluene scintillation mixture.

[1] Abbreviation: Chaps, 3-([3-cholamidopropyl]dimethylammonio)-1-propane sulfonate.

Enzymatic Hydrolysis of Reaction Products

Radioactive glucans produced by Chaps-solubilized enzymes were subjected to enzymatic hydrolysis with purified exocellobiohydrolase from *Trichoderma* (kindly donated by Dr. Driguez, CERMAV, Grenoble, France) or laminarinase from mollusk, which also contains cellulase and α-amylase activities (Ref. L 5144; Sigma). Glucans were suspended in 1 mL of 0.05 M acetate buffer (pH 5) containing 1 mg of enzyme and 0.02% NaN_3 to prevent bacterial contamination. Mixtures were incubated at 40°C from 1 to 20 h. Enzyme digests were chromatographed on Whatman No. 1 paper using propyl alcohol:ethylacetate:water (7:1:2, v/v/v) as solvent. Chromatograms were cut into 1-cm strips for determination of the radioactivity. Standards were run in parallel and detected with 0.3% para-aminohippuric acid in phosphoric acid:80°% ethanol (0.5:100, v/v).

Glucan Synthase Entrapment

Chaps-solubilized enzymes (4 mL) were incubated at 24°C for 20 min in 11-mL assay mixtures for 1,3-β-glucan synthesis. Mixtures were centrifuged for 1 h at 48,000*g* at 4°C through a 5-mL glycerol (20%) cushion. Glucans pelleted with entrapped enzymes were resuspended in 2 mL of Tris-HCl buffer, pH 7.4, then centrifuged for 15 min at 13,000*g* in a microcentrifuge tube. Entrapped enzymes in the pellet were resuspended in 2 mL of buffer and released enzymes from microfibrillar glucans in the supernatant. Both fractions were used for glucan synthase assays and density gradient centrifugation.

Glycerol Density Gradient Centrifugation

Linear gradients (30 mL) were made from 1.025 and 1.113 $g \cdot cm^{-3}$ glycerol in 0.01 M Tris-HCl buffer (pH 7.4) with or without 0.5% Chaps; 2-mL enzyme samples were layered on the gradient and centrifuged at 120,000*g* for 2 or 4 h in a Beckman SW 27 rotor. After centrifugation, 2-mL fractions were collected and assayed for enzyme activities, protein, and refractive index.

SDS-PAGE

SDS-PAGE was carried out on 7.5% slab gel prepared by the procedure of Laemmli (15). Samples were denatured at 100°C for 5 min in 2% SDS, 5% mercapoethanol in Tris-HCl buffer, pH 6.8. Silver staining was performed according to the manufacter's directions (Amersham).

RESULTS

Analysis of the Reaction Products

The [^{14}C]glucans synthesized by solubilized enzymes in the presence of $MgCl_2$ or high substrate concentrations were analyzed for the nature of the β-glycosidic bond configuration. Following exocellobiohydrolase and laminarinase digestions, products were submitted to chromatographic analysis.

Glucans produced at high substrate concentration in the absence of $MgCl_2$ were nearly completely digested by laminarinase to yield glucose (Fig. 1A) but they were not degraded

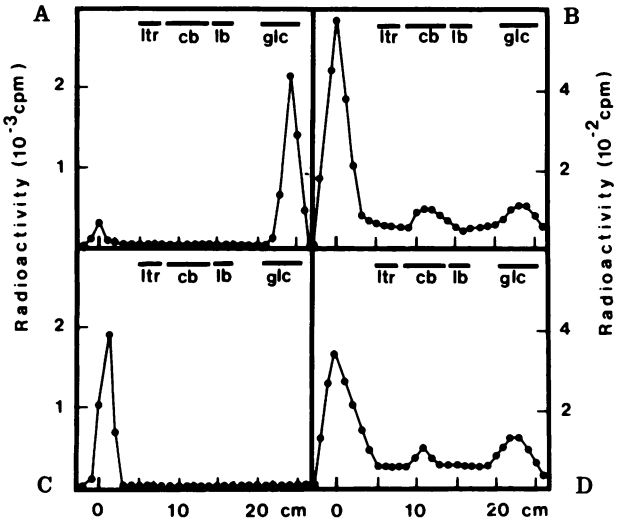

Figure 1. Chromatography of the products of glucan hydrolysis. Chromatographies on Whatman No. 1 paper; propanol:ethyl acetate:water (7:1:2) as solvent. A and C, Glucans produced in the presence of 1.2 mM UDP-Glc without $MgCl_2$ were hydrolyzed by laminarinase (A) and exocellobiohydrolase (C). B and D, Glucans produced in the presence of 0.9 μM UDP-Glc and 15 mM $MgCl_2$ were hydrolyzed by laminarinase (B) and exocellobiohydrolase (D). In the controls, the radioactive products remained at the origin of the chromatograms giving profiles similar to C. glc, glucose; lb, laminaribiose; ltr, laminaritriose; cb, cellobiose.

by the exocellobiohydrolase (even after 20-h incubation), and the polymers remained at the origin of the chromatograms (Fig. 1C). This indicates that 1,3-β-glucans are synthesized in this assay condition.

On the other hand, products synthesized in the presence of Mg^{2+} at low substrate concentration were hydrolyzed by the exocellobiohydrolase, and cellobiose and glucose were detected on chromatograms (Fig. 1D). Laminarinase, which also contains cellulase activity (Ref. L 5144, Sigma), released glucose and cellobiose (Fig. 1B). The efficiency of exocellobiohydrolase and the presence of the 1,4-β dimer of glucose, *i.e.* cellobiose, on chromatograms indicate that 1,4-β-glucans are produced in this assay condition.

These results are in agreement with our previous findings (7, 9) obtained with membrane-bound enzymes, which indicated that the nature of the glucans synthesized is dependent on substrate concentration and on the presence of $MgCl_2$:1,3-β-glucans being synthesized at high UDP-Glc concentration and 1,4-β-glucans formed in the presence of $MgCl_2$ and low UDP-Glc concentration. Recently a 1,4-β-glucan synthase system of *Dictyostelium,* similar to the *Saprolegnia* system, has been described (1). In this cellular slime mold, as in the fungal system, the glucan synthase activity is dependent upon $MgCl_2$ and cellobiose and inhibited by $CaCl_2$.

Glycerol Density Gradient of Solubilized Enzymes

Membrane-bound glucan synthases have proven difficult to purify because these enzymes are very unstable. However, after solubilization with Chaps (5 $mg \cdot mL^{-1}$ at the final con-

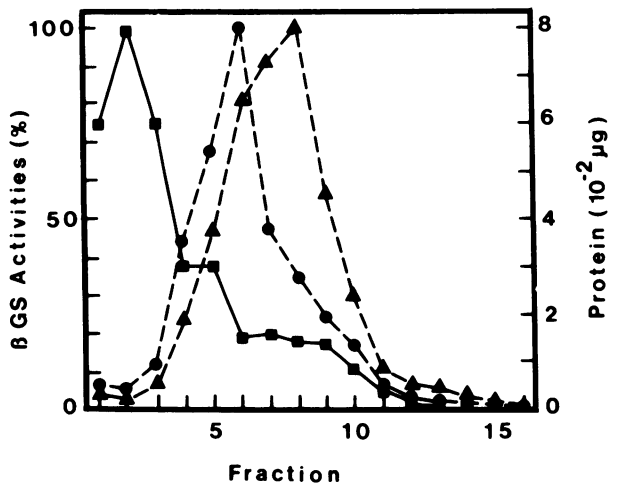

Figure 2. Glycerol gradient centrifugation of Chaps-solubilized glucan synthases. Membrane-bound enzymes solubilized with Chaps (5 $mg \cdot mL^{-1}$) were layered onto 1.025–1.110 $g \cdot cm^{-3}$ linear glycerol gradient and centrifuged for 4 h at 120,000*g*. Fraction 1, top of the gradient. Fraction 16, bottom of the gradient. Results are expressed as the percentage of the maximal value found in a fraction of the gradient. Maximal values were 9.1 nmol of glucose incorporated during 5 min of reaction for 100 µL of enzyme for 1,3-β-glucan synthase (GS) and 3.6 pmol of glucose incorporated during 5 min of reaction for 100 µL of enzyme for 1,4-β-glucan synthase. ■, protein; ●, 1,3-β-glucan synthase activity; ▲, 1,4-β-glucan synthase activity.

centration) the enzymes retained a high activity, and 50% of the total enzymatic activities were recovered in the soluble fraction. The behavior of these enzymes during velocity sedimentation in glycerol gradient is described in Figure 2. Glucan synthase activities were assayed in the presence of $MgCl_2$ or high substrate concentration leading to the synthesis of 1,4-β-glucans or 1,3-β-glucans (9). 1,3-β-Glucan and 1,4-β-glucan synthase activities sedimented as sharp peaks ahead of the bulk of solubilized proteins. After 4-h centrifugation at 120,000*g*, these enzymes were repeatedly separated and recovered at the respective densities of 1.048 and 1.06. This indicates that the two enzymatic complexes are of different high mol wt. The folds of purification measured at the peak of sedimentation were, respectively, 7 and 50 for 1,3-β-glucan and 1,4-β-glucan synthases. When $MgCl_2$, an essential cofactor for 1,4-β-glucan synthase activity was included in the glycerol gradient, enzyme recovery was not improved. Moreover, the enzymes sedimented as a broad peak and 1,3-β-glucan synthase activity, which is inhibited by $MgCl_2$ (7), was very low.

The fractions collected in the glycerol gradient without $MgCl_2$ were analyzed by SDS-PAGE (Fig. 3). Proteins that did not enter the glycerol gradient (Fig. 3, lines 2, 3) exhibited a polypeptide profile very similar to those of the membranes and of the solubilized fractions. Polypeptide composition of the fractions richest in glucan synthase activities (Fig. 3, lines 6–8) were simpler and enriched in subunits of 34, 48, 50, and 65 kD. However, no significant differences were obtained between the peaks of 1,3-β-glucan synthases (Fig. 3, line 6) and 1,4-β-glucan synthases (Fig. 3, line 8).

Separation of the enzyme activities was also achieved when Chaps-solubilized enzymes were centrifuged on glycerol gradients containing 5 $mg \cdot mL^{-1}$ Chaps. After 2-h centrifugation, 1,3-β-glucan synthase activity was recovered as a sharp peak ahead of the solubilized proteins. 1,4-β-Glucan synthases were found at a higher density and in the pellet (Fig. 4A). After 4-h centrifugation, 1,3-β-glucan synthases were found at a high density clearly separated from 1,4-β-glucan synthases, which were mainly collected in the pellet (Fig. 4B).

SDS-PAGE analysis was performed on gradient fractions isolated after 4-h centrifugation (Fig. 5). 1,3-β-Glucan synthase fractions were characterized by a 48- to 50-kD doublet and 65- and 100-kD polypeptides. The occurrence of these polypeptides among the fractions correlated with 1,3-β-glucan synthase activity distribution in the gradient (Fig. 5). The pellet that contained both enzyme activities was enriched in polypeptides of 42, 48, 50, and 55 kD.

Enzyme Entrapment in the Reaction Products

We (9) have previously shown that membrane-bound or solubilized enzymes synthesize *in vitro* microfibrillar glucans. Following incubation in UDP-Glc and low-speed centrifugation, EM examination showed that globular structures of 10-nm diameter were associated with single microfibrils and could represent the glucan synthase complexes (9). Thus, cosedimentation of glucans and enzymes may provide a

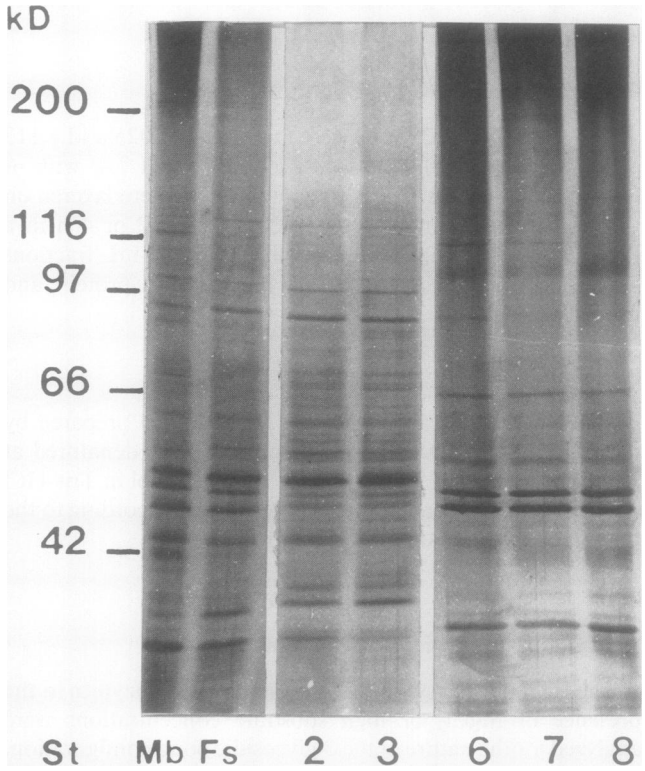

Figure 3. SDS-PAGE analysis of membrane, solubilized enzymes, and gradient fractions of Figure 2. Each lane contained 3 µg of protein stained with silver. Mb, membranes; Fs, solubilized enzymes; 2, 3, 6, 7, 8, glycerol gradient fractions.

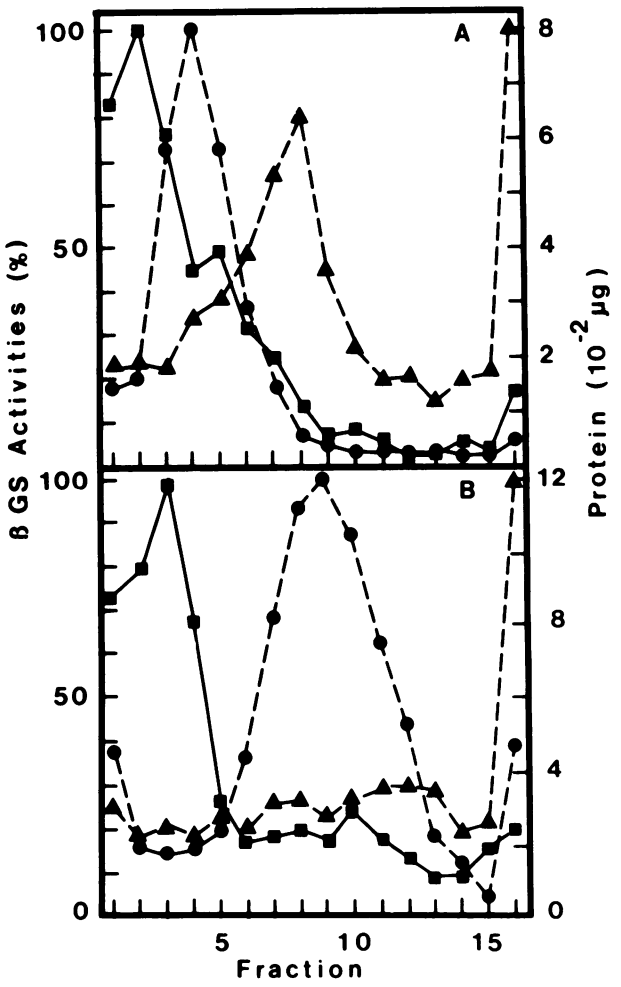

Figure 4. Centrifugation of Chaps-solubilized enzymes in a glycerol gradient containing 0.5% Chaps. Two milliliters of solubilized enzyme preparation were layered onto 1.025–1.110 $g \cdot cm^{-3}$ linear glycerol gradient and centrifuged for 2 h (A) and 4 h (B) at 120,000*g*. Fraction 1, top of the gradient. Fraction 16, bottom of the gradient. Results are expressed as the percentage of the maximal value in a fraction of the gradient. Maximal activities for 1,3-β-glucan synthase (GS) were 26.7 pmol of glucose incorporated during 5 min of reaction for 100 µL of enzyme in gradient A and 12.8 pmol of glucose incorporated during 5 min of reaction for 100 µL of enzyme in gradient B. Maximal activities for 1,4-β-glucan synthase were 7.3 pmol of glucose incorporated during 5 min of reaction for 100 µL of enzyme in gradient A and 15.7 pmol of glucose incorporated during 5 min of reaction for 100 µL of enzyme in gradient B. ■, Protein; ●, 1,3-β-glucan synthase activity; ▲, 1,4-β-glucan synthase activity.

means to isolate glucan synthase. Nonspecific precipitations of 1,3-β-glucan synthases from higher plants occur during incubation of the solubilized enzymes in the absence of substrate (10). This sedimentation is increased by the addition of cation to the solubilized enzymes (Delmer D. P., personal communication). Chaps-solubilized enzymes from *Saprolegnia* were incubated for 30 min in the presence of 15 mM $MgCl_2$ and in the absence of UDP-Glc, then centrifuged at 13,000*g* for 15 min. Three percent of the 1,3-β-glucan synthase activity was recovered in the pellet. But when UDP-Glc was added to the enzymes, more than 20% of the enzymatic activity was sedimented, indicating a product entrapment of the enzymes. When solubilized enzymes were incubated without substrate and without $MgCl_2$, centrifugation did not pellet glucan synthases, indicating that cations such as $MgCl_2$ induced enzyme aggregation. To avoid or reduce this aspecific precipitation, product entrapments were performed in conditions of 1,3-β-glucan synthesis, *i.e.* in the absence of $MgCl_2$. Chaps-solubilized enzymes were incubated in the presence of high UDP-Glc concentration then centrifuged at 13,000*g* for 15 min after various times of incubation. The amounts of sedimentable radioactive glucans increased during the course of incubation. After 1-h incubation, 70% of the glucan produced in the assays could be pelleted. To determine if active enzymes were still associated with the microfibrillar glucans, products were collected by centrifugation after 15-min incubation, then incubated in the enzyme assays. Results described in Figure 6 show that the enzymes entrapped in the aggregated glucans were active, producing 1,3-β-glucans and 1,4-β-glucans. By this product entrapment procedure, 1,3-β-glucan and 1,4-β-glucan synthases were purified respectively, 23- and 80-fold. However, the amount of enzyme recovered was low, because only 18 and 60% of each activity from the solubilized fractions was found to be associated with the fibrillar glucans.

Release of entrapped enzymes was tested by resuspension of the pelleted glucans. Microfibrillar glucans produced *in vitro* were collected by centrifugation (pellet C_1), then resuspended in extraction buffer. The suspension was centrifuged (13,000*g* for 15 min) to separate the glucans (pellet C_2) from detached enzymes (fraction S). Most of the 1,3-β-glucan synthases present in C_1 were recovered in the supernatant (S) with a rate of purification of about 30 (Table I). On the contrary, 1,4-β-glucan synthase activity was still associated with aggregated glucans and collected in the pellet (C_2). This step improved 1,4-β-glucan synthase purification as the fold was about 200. SDS-PAGE analysis of the fractions C_2 and S showed differences in the polypeptide composition (Fig. 7, A and B). C_2, rich in 1,4-β-glucan synthase, showed an enrichment in subunits of 52, 58, and 60 kD. Subunits of 34, 48, and 50 kD were heavily stained in the 1,3-β-glucan synthase fraction (S).

Glycerol Gradient of Entrapped Enzymes

To achieve a better purification, enriched enzymatic fractions obtained after resuspension of the enzymes entrapped in aggregated glucans were centrifuged on glycerol gradients (Fig. 8).

After 4-h centrifugation at 120,000*g* of the fraction S (*i.e.* enzymes released following resuspension of sedimented glucans), 1,3-β-glucan synthase activity was recovered as a sharp peak at the top of the gradient associated with a very low 1,4-β-glucan synthase activity (Fig. 8A). Fraction C_2 (*i.e.* enzymes remaining associated to sedimented glucans) was centrifuged for 30 min at 120,000*g*. Microfibrillar glucans contained in this fraction sedimented through the gradient (Fig. 8B) and enzyme activities were recovered in the pellet (P). 1,4-β-Glucan synthase activity was very high, corresponding to a degree of purification of 370. A weak 1,3-β-glucan synthase activity was still associated with the glucan fibrils. Following

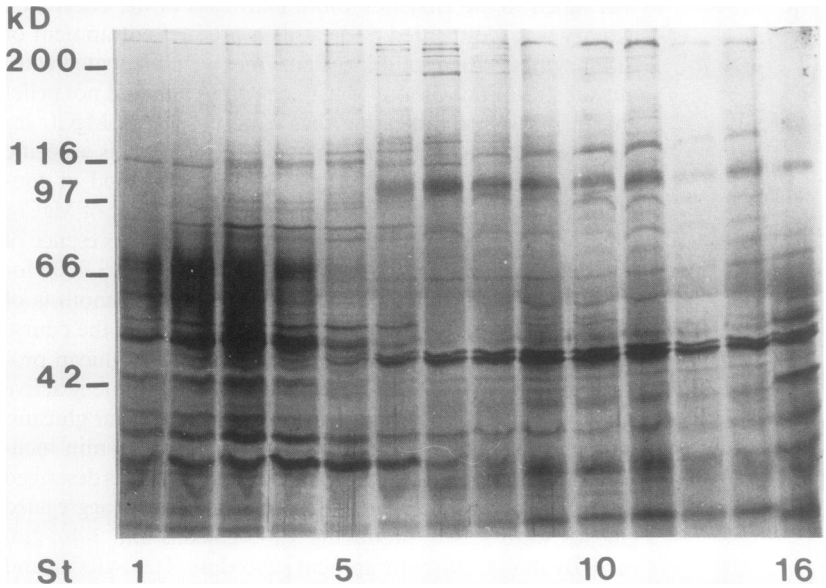

Figure 5. SDS-PAGE analysis of the fractions of gradient B of Figure 4. Each lane contained 3 μg of proteins stained with silver. Lanes 1 to 13, lanes 1 to 13 of the gradient; lane 16, glycerol gradient pellet.

these density gradients, recovery of 1,3-β-glucan and 1,4-β-glucan synthase activities were, respectively, about 4 and 48%. These yields and rates of purification are probably underestimated and could not be reliably quantitated because the enzyme activities were quite unstable over the length of manipulation. We have observed that after 24 h at 4°C, enzyme activities decreased up to 50%.

The enriched enzymatic fractions from glycerol gradients were analyzed by SDS-PAGE. Their protein subunit composition was compared with those of the fractions collected at the different steps of purification. The fraction containing 1,3-β-glucan synthase activity (Fig. 7A, lanes 1 and 2) was characterized by three main polypeptides of 34, 48, and 50 kD, but other polypeptides of 25, 30, 32, 80, 90, and 100 kD were also detected by sensitive silver staining. These minor bands were not detected after staining with Coomassie blue (not shown). The fraction enriched in 1,4-β-glucan synthase was characterized by polypeptides of 52, 58, and 60 kD (Fig. 7B, lane P). This fraction, which also contained a low 1,3-β-glucan synthase activity, exhibited weakly stained bands (34, 48, and 50 kD) characterizing the 1,3-β-glucan synthase activity. The intensity of the typical bands of each enzyme was found to correlate with the enzyme activities at each step of purification. The presence of several subunits in each enriched enzymatic fraction suggests that the enzymes have an oligomeric structure.

DISCUSSION

Many studies have been focused on the mechanism of activation and regulation of 1,3-β-glucan and 1,4-β-glucan synthases (3, 11, 14). A multitude of factors (Ca^{2+}, Mg^{2+}, proteases, nucleotides, phosphatases) have been identified as potential regulators of these enzymes (7, 10, 11, 13, 18, 19). Purified enzymes would allow a new appraisal of the role of these factors. This would also permit determination of their target, enzymes, regulatory proteins, and/or phospholipid environment and also allow an answer to the potential rela-

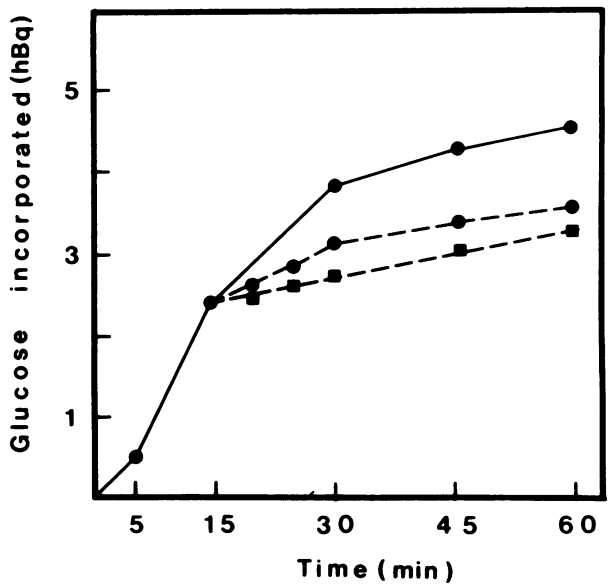

Figure 6. Enzymatic activities of entrapped enzymes in aggregated glucans. Chaps-solubilized enzymes (100 μL) incubated in 1.2 mM UDP-Glc were centrifuged at different times of incubation to measure the sedimentable glucans (●——●). After 15-min incubation, glucans were sedimented at 13,000*g* for 15 min and then resuspended in enzyme assay mixture to measure associated 1,4-β-glucan synthase activity (■ - - - ■) and 1,3-β-glucan synthase activity (● - - - ●).

tionship between 1,4-β-glucan and 1,3-β-glucan synthases, *i.e.* are glucan synthases able to catalyze either cellulose or callose synthesis reversibly, depending on conditions (3)?

Gradient centrifugation of solubilized enzyme has separated *Saprolegnia* 1,3-β-glucan and 1,4-β-glucan synthases into overlapping, but distinct peaks of activity, 1,4-β-glucan synthase exhibiting a higher apparent mol wt. Behavior of glucan synthases during glycerol gradient centrifugation was dependent on the presence of detergent in the gradient. In the absence of Chaps, 1,3-β-glucan synthase remained in the light densities. In the presence of detergent, enzymes entered more easily in the gradient because their lipidic environment was maintained and 1,4-β-glucan synthases were pelleted through gradient containing detergent. SDS-PAGE analysis of the enzyme fractions collected on these two types of gradient showed that polypeptide profiles were similar. 1,3-β-Glucan synthase was characterized by a heavily stained doublet of 48 to 50 kD, and peptides of 65 and 105 kD. This indicates that at least in fungi, two types of enzymes are involved in cell wall polysaccharide synthesis.

Improvement of the enzyme purification using an entrapment procedure and gradient centrifugation permits us to reach a high degree (varying from 200 to 400) of 1,4-β-glucan synthase purification. The rate of purification of 1,3-β-glucan synthase was much lower (≈30). This low value could be explained by the relative instability of the enzymes over the time of experimentation. An interesting observation was that the enzyme of the most purified fraction just entered the glycerol gradient, whereas 1,3-β-glucan synthase solubilized by Chaps treatment of the membranes had a higher apparent mol wt. This may relate to the elimination of contaminating proteins and phospholipids during the procedure of purification, some of which might be essential to maintain full enzyme activity. Their elimination would thus reduce specific enzyme activity leading to the underestimation of the purification degrees.

SDS-PAGE analysis of purified 1,3-β-glucan and 1,4-β-glucan synthases revealed a large difference between their polypeptide profiles. The most purified fractions were characterized by three main polypeptides of different mol wt; 34, 48, and 50 kD for 1,3-β-glucan synthases and 52, 58, and 60 kD for 1,4-β-glucan synthases.

Interestingly, polypeptides of very similar sizes have also been recently identified in several other laboratories. Using photo-affinity labeling, UDP-Glc binding polypeptides of higher plant 1,3-β-glucan synthases have been demonstrated. A polypeptide of 57 kD of red beet has been shown to bind 5-azido-uridine 5′-β-[^{32}P]diphosphate glucose (10, 22) and polypeptides of 50 and 34 kD from cotton fiber were labeled with uridine 5′-β-[^{32}P]diphosphate glucose following UV irradiation (4). Two polypeptides of 67 and 57 kD of the cellulose synthase from *Acetobacter* were also labeled with radioactive substrate (17). By using the substrate analog uridine 5′-β-[^{32}P] diphosphate glucose, a polypeptide of 83 kD from purified cellulose preparation of an other *Acetobacter xylinum* strain was photolabeled (16).

Delmer (3) has proposed that 1,4-β-glucan synthase would be converted by conformational changes to 1,3-β-glucan synthases and that one enzyme would be able to catalyze the synthesis of cellulose or 1,3-β-glucans. In our system, 1,4-β-glucan synthase displays bands on SDS-PAGE larger than 1,3-β-glucan synthase bands. These results may rule out Delmer's hypothesis, at least for *Saprolegnia* enzymes. On the

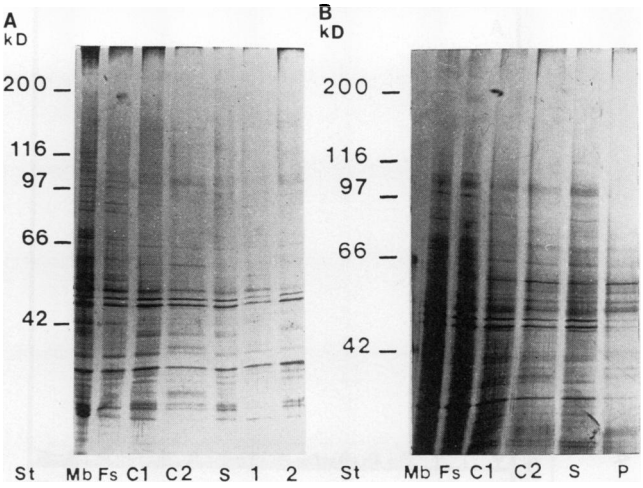

Figure 7. SDS-PAGE analysis of membranes, solubilized enzymes, entrapped enzymes, and gradient fractions from Figure 8, A and B. Each line contained 3 μg proteins stained with silver. Mb, membranes; Fs, Chaps-solubilized enzymes; C1, entrapped enzymes; C2, enzymes associated to aggregated glucans following resuspension of pellet C1; S, enzymes released following resuspension of pellet C1; P, pellet from gradient Figure 8B; 1–2, fractions from gradient Figure 8A.

Table I. *Purification of 1,3-β-Glucan and 1,4-β-Glucan Synthases*

	Total proteins	Activity		Specific activity[d]		Purification fold	
		1,3-β-GS[a,b]	1,4-β-GS[c]	1,3-β-GS	1,4-β-GS	1,3-β-GS	1,4-β-GS
	mg					*– fold*	
Solubilized enzymes	46	2870	187	62	4	1	1
Pellet C1	0.4	520	87	1443	240	23	80
Pellet C2	0.1	38	67	346	609	6	203
Supernatants	0.3	503	42	1676	139	27	46

[a] GS, glucan synthase. [b]Expressed as nmol of glucose incorporated during 5-min incubation. [c]Expressed as pmol of glucose incorporated during 5-min incubation. [d]Expressed as nmol of glucose incorporated/mg of proteins for 1,3-β-GS and pmol of glucose incorporated/mg of proteins for 1,4-β-GS.

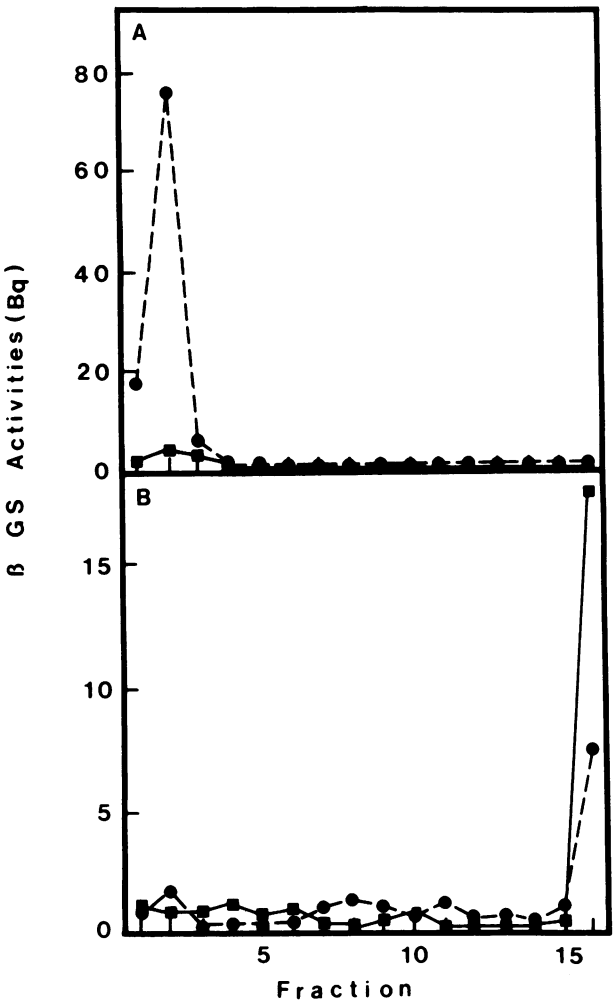

Figure 8. Glycerol gradient centrifugation of enzymes released (A) from aggregated glucans and of enzymes associated (B) to aggregated glucans. Chaps-solubilized enzymes were incubated in 1.2 mM UDP-Glc, then entrapped enzymes were collected by centrifugation at 48,000*g* for 1 h. The pellet (C1) was resuspended in extraction buffer then centrifuged at 13,000*g* for 15 min giving two fractions: enzymes released in the supernatant (S), and enzymes still associated with the glucans (C2). Each fraction was layered onto 1.025 to 1.110 $g \cdot cm^{-3}$ glycerol gradient. Fraction S was centrifuged for 4 h at 120,000*g* (A). Fraction C2 was centrifuged 30 min at 120,000*g* (B). Fraction 1, top of the gradient. Fraction 16, bottom of the gradient; ●, 1,3-β-glucan synthase (GS) activity; ■, 1,4-β-glucan synthase activity.

other hand, they may favor the hypothesis of Girard and Maclachlan (11), who have proposed that a limited protease action would convert 1,4-β-glucan synthase to 1,3-β-glucan synthase. However, the fact that 1,3-β-glucan synthase units originate from 1,4-β-glucan synthase subunits remains to be demonstrated.

In our system, the enzymes may have an oligomeric structure composed of different subunits as several polypeptides characterized each of the purified enzymes. The cellulose synthase from *Acetobacter* is also an oligomeric protein as the purified enzyme (350-fold) contains three major (Coomassie stain) polypeptides (16, 17).

A common organization of glucan synthases implying a common mechanism of synthesis seems to be a feature of β-glucan polymerases of the different systems.

To comprehend the catalytic and regulatory mechanism of the enzymes, isolation and characterization of each constitutive subunit must be undertaken. The purification protocol described in this paper combined with photo-affinity labeling of substrate binding sites (10, 16, 17) and to monoclonal antibody techniques (16) would provide the tools to investigate the properties of polysaccharide synthases.

ACKNOWLEDGEMENTS

The authors are grateful to Gordon Maclachlan for useful comments on this manuscript.

LITERATURE CITED

1. **Blanton RL, Northcote DH** (1990) A 1,4-β-D-glucan synthase system from *Dictyostelium discoideum*. Planta **180:** 324–332
2. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
3. **Delmer DP** (1987) Cellulose biosynthesis. Annu Rev Plant Physiol **38:** 259–290
4. **Delmer DP, Salomon M** (1989) Identification of polypeptides involved in plant β glucan synthesis (Abstract 36). *In* Proceedings of the Fifth Cell Wall Meeting, Edinburgh
5. **Eiberger LL, Wasserman PB** (1987) Partial purification of digitonin-solubilized β glucan synthase from red beet root. Plant Physiol **83:** 982–987
6. **Fèvre M** (1979) Digitonin solubilization and protease stimulation of β glucan synthetases of *Saprolegnia*. Z Pflanzenphysiol **95:** 129–140
7. **Fèvre M** (1983) Nucleotide effects on glucan synthesis activities of particulate enzymes from *Saprolegnia*. Planta **159:** 130–135
8. **Fèvre M, Girard V, Nodet P** (1988) Cellulose and β glucan synthesis in *Saprolegnia*. *In* Kuhn, ed, Biochemistry of Cell Walls and Membranes in Fungi. Springer-Verlag, Heildelberg, pp 97–107
9. **Fèvre M, Rougier M** (1981) 1,3-β and 1,4-β glucan synthesis by membrane fractions from the fungus *Saprolegnia*. Planta **157:** 232–241
10. **Frost DJ, Read SM, Drake RR, Haley BE, Wasserman BP** (1990) Identification of the UDP-glucose-binding polypeptide of callose synthase from *Beta vulgaris* L. by photoaffinity labeling with 5-azido-UDP-glucose. J Biol Chem **265:** 2162–2167
11. **Girard V, Maclachlan GA** (1987) Modulation of pea membrane 1,3-β-glucan synthase activity by calcium, polycation, endogenous protease activity and protease inhibitor. Plant Physiol **85:** 131–136
12. **Hayashi T, Read SM, Russel J, Thelen M, Lin FC, Brown RM, Delmer DP** (1987) UDP-glucose 1,3-β-glucan synthases from mung bean and cotton. Differential effects of Ca^{2+} and Mg^{2+} on enzyme properties and on macromolecular structure of the glucan product. Plant Physiol **83:** 1054–1062
13. **Kang MS, Elango N, Mattia E, Au-Young J, Robbins PN, Cabib E** (1984) Isolation of chitin synthase from *Saccharomyces cerevisiae*. Purification of an enzyme by entrapment in the reaction product. J Biol Chem **259:** 14966–14972
14. **Kauss H** (1985) Callose biosynthesis as a Ca^{2+} regulated process and possible relations to the induction of other metabolic changes. J Cell Sci **2** (Suppl)**:** 89–103
15. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

16. **Lin FC, Brown RM Jr, Drake RD, Haley BE** (1990) Identification of the uridine 5′-diphosphoglucose (UDP-Glc) binding subunit of cellulose synthase in *Acetobacter xylinum* using the photoaffinity probe 5-azido-UDP-Glc. J Biol Chem **265:** 4782–4784
17. **Mayer R, Ross P, Weinhouse H, Amikam D, Volman G, Ohana P, Benziman M** (1989) The polypeptide substructure of bacterial cellulose synthase and its occurrence in higher plants. (Abstract 38). *In* Proceedings of the Fifth Cell Wall Meeting, Edinburgh
18. **Nodet P, Grange G, Fèvre M** (1988) Dot-blot assays and their use as a direct antigen binding method to screen monoclonal antibodies to 1,4-β and 1,3-β glucan synthases. Anal Biochem **174:** 662–665
19. **Read SM, Delmer DP** (1987) Inhibition of mung bean UDP-glucose 1,3-β-glucan synthase by UDP-pyridoxal. Evidence for an active site amino group. Plant Physiol **85:** 1008–1015
20. **Ross P, Aloni Y, Weinhouse H, Michaeli D, Weinberger-Ohana P, Mayer R, Benziman M** (1986) Control of cellulose synthesis in *Acetobacter xylinum*. A unique guanyl oligonucleotide is the immediate activator of the cellulose synthase. Carb Res **149:** 101–117
21. **Saugy M, Farkas W, Maclachlan G** (1988) Phosphatases and phosphodiesterases interfere with 1,3-β-D glucan synthase activity in pea epicotyl membrane preparation. Eur J Biochem **177:** 135–138
22. **Wasserman PB, Frost DJ, Wu A, Read SM, Drake RR, Haley BE** (1989) Identification of the UDP-binding polypeptide of 1,3-β glucan synthase of higher plants by photoaffinity labeling with 5-azido-UDPG (Abstract 35). *In* Proceedings of the Fifth Cell Wall Meeting, Edinburgh